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Synthesis of DNA via deoxynucleoside H-phosphonate intermediates

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ABSTRACT

Deoxynucleoside H-phosphonates are used in the chemical synthesis of deoxyoligonucleotides up to 107 bases in length. The biological activity of the synthetic DNA is assessed by cloning into M13 and sequencing. An improved synthesis of protected deoxynucleoside H-phosphonates is also described.

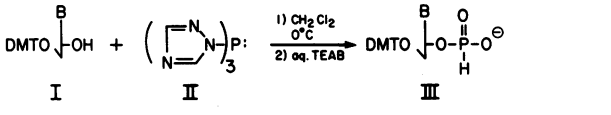
INTRODUCTION

Activation of deoxynucleoside H-phosphonates with acylating agents generates a highly reactive and selective agent for the formation of H-phosphonate diesters (1). Recently the chemical synthesis of tetracontathymidylic acid (T_{40}) was accomplished using trimethylacetyl choride as the activator (1). Described herein is the use of deoxynucleoside H-phosphonate intermediates in the synthesis of DNA containing the four common deoxynucleotides utilizing a rapid, simple and reagent efficient synthetic protocol. Deoxyoligonucleoside H-phosphonate diesters are stable to the reaction conditions of DNA synthesis and are converted to the native deoxyoligonucleotide phosphate diesters by aqueous I_2 oxidation. Also described is an improved synthesis of protected deoxynucleoside H-phosphonates.

MATERIALS AND METHODS

The previously reported syntheses of deoxynucleoside H-phosphonates (IIIa-d) (2,3) are troublesome with variable yields, therefore we have developed an improved synthesis of IIIa-d. The reaction of protected deoxynucleoside (Ia-d, Scheme 1) with tris (1,2,4-triazoyl) phosphite (II) followed by hydrolysis yielded IIIa-d. II was generated *in situ* from phosphorus trichloride (PCl_3), 1,2,4-triazole and N-methyl morpholine in anhydrous methylene chloride (CH_2Cl_2). Deoxynucleoside H-phosphonates

Scheme 1



(IIIa-d) are stable, hygroscopic solids that can be stored at room temperature.

Preparation of 5'-Dimethoxytrityl-3'-Thymidine H-Phosphonate (IIIa)(Scheme 1)

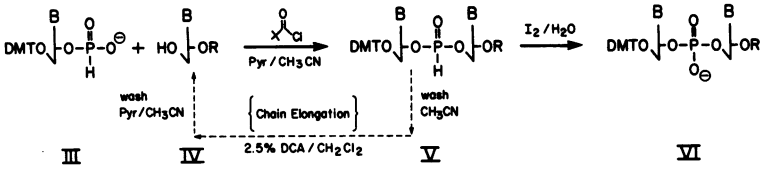
To a stirred solution of PCl_3 (75mmole) and N-methyl morpholine (750mmole) in 750 ml anhydrous CH_2Cl_2 was added 1,2,4-triazole (250mmole) at room temperature. After 30 min. the reaction mixture was cooled to 0°C and 5'-Dimethoxytrityl thymidine (Ia, 15mmole, dried by co-evaporation from CH_3CN) in 200 ml anhydrous CH_2Cl_2 was added dropwise over 20 min., stirred for 10 min, poured into 600 ml of 1.0M aqueous triethylammonium bicarbonate (TEAB, pH 8.5), shaken and separated. The aqueous phase was extracted with 200 ml CH_2Cl_2 and the combined organic phase was dried over Na_2SO_4 and evaporated to a foam. Silica gel column chromatography (2 percent Et_3N/CH_2Cl_2 → 2 percent Et_3N/10 percent MeOH/CH_2Cl_2) followed by TEAB extraction and evaporation yielded 5'-dimethoxytrityl-3'-thymidine H-phosphonate (IIIa) in 90 percent yield. All other deoxy-

Table 1

Nucleoside H-phosphonate	^{31}P NMR[1] Chemical Shift	J(P-H)	Yield[2] (Percent)
thymidine (IIIa)	-0.3 ppm	605 Hz	90
deoxyadenosine (IIIb)	-0.3 ppm	600 Hz	85
deoxycytidine (IIIc)	-0.3 ppm	603 Hz	86
deoxyguanosine (IIId)	-0.6 ppm	605 Hz	77

1) ^{31}P NMR spectra were obtained in an anhydrous Pyr/CH_3CN (1/1) solution and chemical shifts are reported relative to 5 percent phosphoric acid/D_2O (external standard).
2) Isolated yield based upon protected deoxynucleoside (Ia-d).

Scheme 2



nucleoside H-phosphonates were prepared by the same procedure, isolated yields and ^{31}P NMR data are reported in Table 1.

Synthesis of Deoxyoligonucleotides

Syntheses were performed with a Biosearch Model 8600 DNA synthesizer. Trimethylacetyl chloride (pivaloyl chloride) was distilled at atmospheric pressure and stored under Argon (Ar). Pyridine (Pyr) was distilled from p-toluenesulfonyl chloride, then freshly distilled from calcium hydride. Acetonitrile (CH_3CN) was dried over activated 3Å molecular sieves. Deoxynucleoside H-phosphonates (IIIa-d) were dried by co-evaporation from anhydrous CH_3CN and reconstituted in anhydrous Pyr/CH_3CN (1/1). Syntheses were performed on control pore glass (0.1 μmole scale) using the following protocol.

Synthetic Cycle (Scheme 2)

1) Wash - anhydrous CH_3CN (45 sec).
2) Deblock - 2.5 percent dichloroacetic acid (DCA)/CH_2Cl_2 (1 min.).
3) Wash - anhydrous Pyr/CH_3CN (45 sec).
4) Couple - 10 m$\underline{M}$ deoxynucleoside H-phosphonate (Ia-d), 50 m$\underline{M}$ pivaloyl chloride in anhydrous Pyr/CH_3CN (1/1) (1.5 min).
5) Repeat step 1 until the nucleotide sequence is complete.
6) Deblock - 2.5 percent DCA/CH_2Cl_2 (1 min).
7) Oxidize H-phosphonate DNA with: 1) 0.1 $\underline{M}$ I_2 in Pyr/NMI/H_2O/THF (5/1/5/90) (2.5 min), 2) 0.1 $\underline{M}$ I_2 in Et_3N/H_2O/THF (5/5/90) (2.5 min). The DNA was removed from the polymer, deprotected (conc. NH_4OH, 55°C, 5 hrs.) and evaporated.

RESULTS AND DISCUSSION

Deoxynucleoside H-phosphonates (IIIa-d) are used for the complete synthesis of deoxyoligonucleotides (DNA) up to 107 bases in length. The synthetic protocol described above (see Materials and Methods) uses a minimal

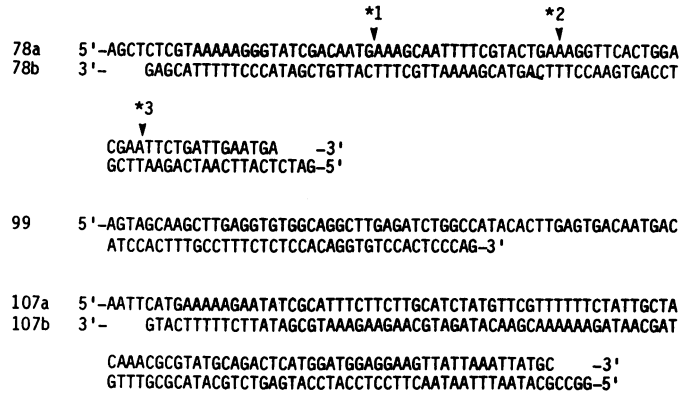


Figure 1. Sequences of synthesized deoxyoligonucleotides.

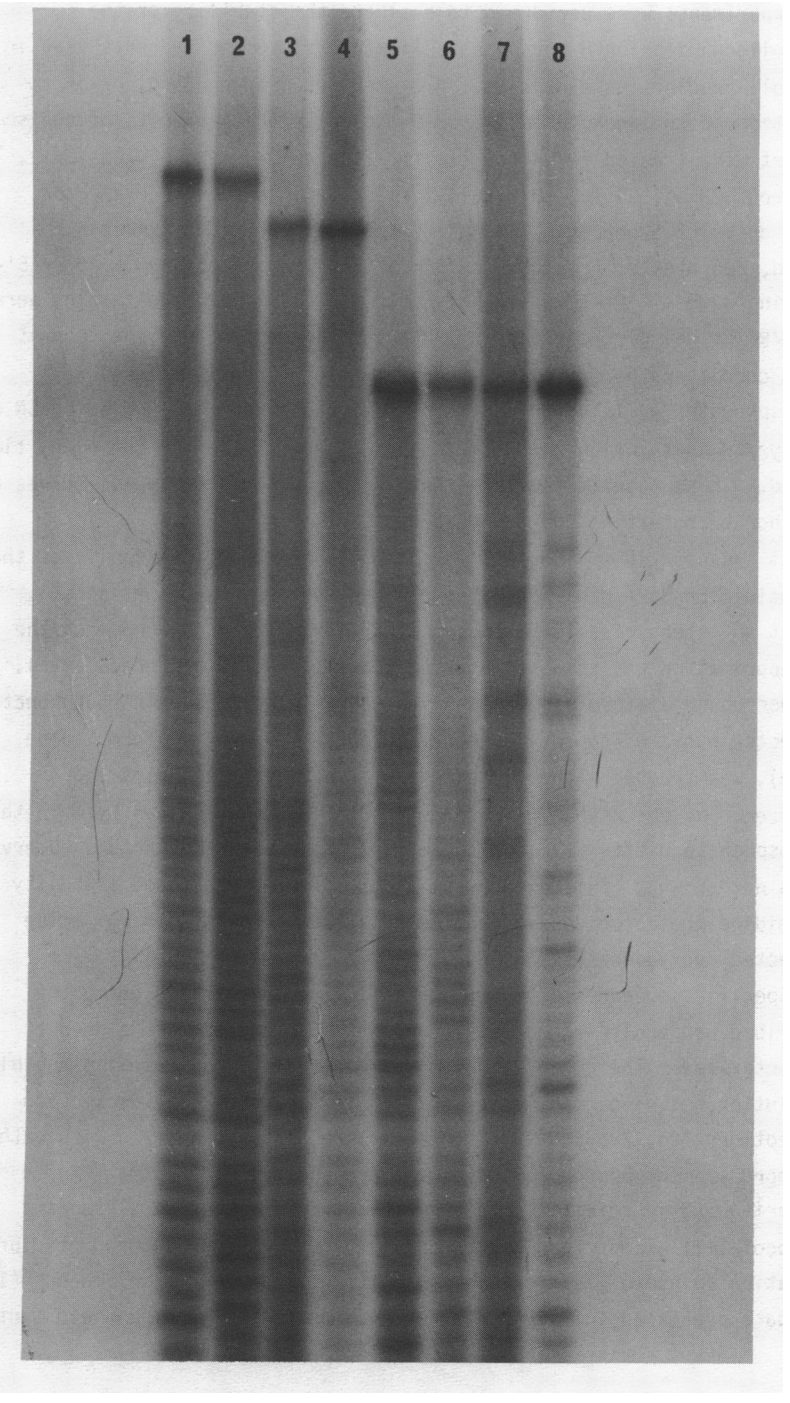
amount of deoxynucleoside H-phosphonate (2.5 mg/coupling) in a simplified and rapid procedure (4 min/cycle). The internucleoside phosphorus is protected from unwanted side reactions by its oxidation state and the native phosphate diester is generated after synthesis by aqueous I_2 oxidation.

Oxidation of a polymer bound polynucleoside H-phosphonate (V) with 0.1M I_2 in a Pyr/H_2O/THF (5/5/90) solution has been inadequate for consistent results. The aqueous I_2 oxidation of dialkyl H-phosphonates is subject to general base catalysis (4) and we have determined that addition of N-methyl imidazole (NMI) or stronger bases (i.e. N-methyl morpholine or triethylamine (Et_3N)) increases the rate of oxidation and is essential for the oxidation of long deoxyoligonucleoside H-phosphonates (V, >40 bases). Dialkyl H-phosphonates are susceptible to alkaline hydrolysis (5) and we have observed that the aqueous oxidation of polynucleoside H-phosphonates (V) using Et_3N is complicated by competitive hydrolysis. The I_2 oxidation of long deoxyoligonucleoside H-phosphonates (V, >40 bases) first with a weakly basic solution (Pyr/NMI) followed by oxidation with a strongly basic solution (Et_3N) gives the highest yield of product deoxyoligonucleotide (VI). Additionally we have found that an aqueous I_2 solution, after sitting for several days, fails to oxidize deoxyoligonucleoside H-phosphonates (V). An aqueous I_2 solution disproportionates to IO_3^- and I^- (4,6) and an alkaline IO_3^- solution will not oxidize a dinucleoside H-phosphonate (V) (7), therefore it is necessary to prepare separate solutions [A=0.2M I_2/THF, B=Base/H_2O/THF (1/1/8)] and mix immediately prior to use for the consistent and rapid oxidation of deoxyoligonucleoside H-phosphonates (V).

Experiments were performed to evaluate the stability of the deoxyoligonucleoside H-phosphonate linkage (V) to the reagents used in deoxyoligonucleotide synthesis. A 24-mer was prepared and, prior to oxidation of the deoxynucleoside-H-phosphonate (V), aliquots of the solid support were treated for 4 hours with: 1) anhydrous Pyr/CH_3CN (1/1), 2) anhydrous CH_3CN, 3) 100 m$\underline{M}$ pivaloyl chloride in anhydrous Pyr/CH_3CN (1/1) and 4) 2.5 percent DCA/CH_2Cl_2, washed with CH_3CN, oxidized, washed, deprotected (conc. NH_4OH/5 hr./55°C) and evaporated. After 5'-end labeling with γ-^{32}P-ATP using polynucleotide T4 kinase the samples were analyzed by gel electrophoresis and autoradiography (data not shown). Under these conditions no detectable degradation of the polynucleoside H-phosphonate (V) is observed, but addition of 1 percent H_2O to CH_3CN or the Pyr/CH_3CN solution leads to detectable degradation in the 4 hr. time period. These results indicate that the H-phosphonate linkage serves as a phosphorus protecting group throughout synthesis.

It has been shown that a 3' or 5'-phosphate triester stabilizes the glycosidic bond of deoxyadenosine to acid mediated depurination (8). The effect an internucleoside H-phosphonate linkage (V) would have on the rate of depurination was assessed by a previously described procedure (9). A polymer bound 3'-thymidine-5'-deoxyadenosine dinucleoside H-phosphonate, protected has a N^6-benzoyl amide (Vb,a) or N^6-diisobutyl formamidine (Ve,a), was treated with 2.5 percent DCA/CH_2Cl_2 for 24 hours and analyzed. No detectable difference in rate of depurination between the H-phosphonate diester (V) and the methyl phosphate triester was observed (data not shown). This experiment also confirmed the added stability of the N^6-amidine protected deoxyadenosine relative to the N^6-benzoyl amide protected deoxyadenosine (9,10).

Specific sequences of DNA (Figure 1) were synthesized using the described protocol (see Materials and Methods) and the crude product characterized. The audioradiogram derived from 5'-end labeling and high resolution gel electrophoresis is shown in Figure 2. The deoxyoligonucleotides 78a, 78b and 99 (Figure 1) were also synthesized with methoxy diisopropylamino phosphoramidites and are included in Figure 2 for comparison. The amidite syntheses were carried out using a standard protocol (11) except that a 30 second aqueous wash was incorporated prior to oxidation to hydrolyze possible phosphite-heterocyclic base adducts (12). The data presented in Figure 2 clearly demonstrate the high yield synthesis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of product deoxyoligonucleotides using deoxynucleoside H-phosphonate intermediates.

Deoxyoligonucleotides 78a and 78b were further characterized by cloning into M13 and sequencing. The DNA was purified by gel electrophoresis, the product band identified by U.V. shadowing (Figure 3), cut out, eluted and precipitated from ethanol. The complementary 78-mers, upon hybridization, yield a duplex with a 4 base overlap on each 5'-end. The duplex bears the ends produced by digestion of the HindIII and BamHI restriction enzymes. Direct ligation of 20ng of duplex into 20ng of M13 vector (M13mp18), previously digested with HindIII and BamHI, followed by transformation of competent E. coli (JM101) and plating yielded white plaques. A comparison ligation and transformation of an equal amount of 78-mer duplex synthesized by the phosphoramidite method gave a similar number of white plaques. This demonstrates comparable biological activity for the two duplexes, one synthesized from H-phosphonate intermediates and the other synthesized from phosphoramidites. Ten M13 plaques, from both methods, were sequenced by a standard procedure (13). Seven clones, derived from H-phosphonates, were identical to the desired sequence. One clone contained a deletion of deoxyadenosine at position *2 (Figure 1). Two clones contained one base insertions: deoxyguanosine at position *1 and deoxyadenosine at position *3 (Figure 1). All ten amidite clones contained sequences which were identical to the desired sequence. Using a similar procedure the duplex derived from deoxyoligonucleotides 107a and 107b were cloned and both clones that were sequenced were identical to the desired sequence.

The result of an insertion or deletion, in a small percentage of duplexes derived from H-phosphonates, is not surprising in view of the audioradiogram shown in Figure 2. Examination of the product band reveals a major 78-mer band flanked by minor, but observable, amounts of a 79-mer and 77-mer which co-purify with the major band during preparative gel electrophoresis (Figure 3). Such products would be expected to clone into M13 if they are a result of random internal insertions or deletions. An explanation, although not confirmed, for the observed insertions is that a

Figure 2. Autoradiogram of 8 percent polyacrylamide sequencing gel derived from ^{32}P 5'-phosphorylated unpurified deoxyoligonucleotides. The lanes are as follows: Lane 1, sequence 107a; Lane 2, sequence 107b; Lane 3, sequence 99 synthesized using H-phosphonates; Lane 4, sequence 99 synthesized using phosphoramidites; Lane 5, sequence 78a synthesized using H-phosphonates; Lane 6, sequence 78b synthesized using H-phosphonates; Lane 7, sequence 78a synthesized using phosphoramidites; Lane 8, sequence 78b synthesized using phosphoramidites.

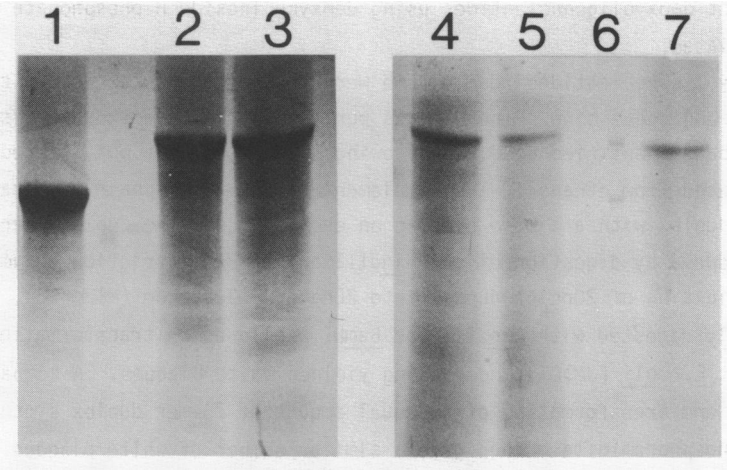

Figure 3. U.V. shadowing photograph of the 10 percent polyacrylamide denaturing gel (7M urea) of deoxyoligonucleotides derived from H-phosphonates. The lanes are as follows: Lane 1, 58-mer size standard; Lane 2, sequence 78a; Lane 3, sequence 78b; Lane 4, sequence 107a; Lane 5, sequence 107b; Lane 6, 78 and 99-mer size standard; Lane 7, sequence 99.

small amount of detritylation occurs during the coupling reaction. This would lead to trace dimer addition during that step of the synthesis.

A distinguishing feature of all the clones sequenced is the lack of point mutations. This implies a lack of stable modification of the purine or pyrimidine rings during synthesis. This is an interesting result in light of the recent efforts to introduce O^6 protection of deoxyguanosine in deoxyoligonucleotide synthesis to prevent base modification (14,15). At this time the sequence fidelity of H-phosphonate DNA is lower than that produced by phosphoramidites; however, this disadvantage is offset by a simpler synthetic cycle and significantly lower concentration of deoxynucleoside H-phosphonate required for synthesis.

CONCLUSIONS

Deoxyoligonucleoside H-phosphonates (IIIa-d) have been used in the direct synthesis of polynucleoside H-phosphonate diesters (V). The internucleoside H-phosphonate is stable to the reaction conditions of DNA synthesis and converted to the native phosphate diester by aqueous I_2 oxidation. This method has been used for the synthesis of DNA up to 107 bases in length using a rapid, simple and reagent efficient synthetic protocol. The resulting synthetic DNA has been shown to be biologically

active with high sequence fidelity. The results presented above clearly demonstrate the usefulness of deoxynucleoside H-phosphonates (IIIa-d) in the chemical synthesis of DNA.

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